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Assessing the effect of oxygen and microbial inhibitors to optimize ferricyanide-mediated BOD assay

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ABSTRACT

Methods for short-term BOD analysis (BOD_{st}) based on ferricyanide mediator reduction have succeeded in overcoming some problems associated with the standard BOD test analysis (BOD_5) such as long-term incubations (5 days), the need to dilute samples and low reproducibility. Here we present a bioassay where a *Klebsiella pneumoniae* environmental strain successfully reduces ferricyanide without de-aeration of the samples with linear BOD_5 ranges between 30 and 500 mg L^{-1} or 30 and 200 mg L^{-1}, using glucose-glutamic acid solution (GGA) or OECD standards respectively. We further propose a new assay termination solution that allows higher reproducibility and standardization of the cell-based assay, employing formaldehyde (22.7 g L^{-1}) or other compounds in order to stop ferricyanide reduction without affecting the amperometric detection and therefore replace the centrifugation step normally used to stop microbial-driven reactions in ferricyanide-mediated bioassays. These improvements led to an accurate determination of real municipal wastewater samples.

1. Introduction

BOD is widely used as an indicator of the amount of easily biodegradable organic matter in water samples. The standard method for evaluating BOD involves measurements of the amount of dissolved oxygen consumed after 5 days of incubation under specific conditions proposed by the *Standard methods for the examination of water and wastewater* [1] in a microbially inoculated sample (with either a commercial blend or an indigenous population).

A standard glucose-glutamic acid solution (GGA) is frequently used to verify the methodological protocols and the viability of indigenous populations. The known BOD_5 value of a GGA solution containing 150 mg L^{-1} of glutamic acid and 150 mg L^{-1} of glucose is 198 ± 31 mg L^{-1} (expressed as mg L^{-1} of consumed O_2). Synthetic sewage found in the *OECD 303A test guideline* [2] is also a standard employed to assess the biodegradability or the removal of substances in activated sludge systems.

However, the BOD_5 standard method presents several drawbacks: the method is time-consuming and depends heavily on the experience of operators to achieve reproducible and reliable results. Since it takes 5 days for BOD determination, this method is not suitable for process control and real-time monitoring. In the case of a pollution event in a natural ecosystem or an overload in a wastewater treatment plant, results would be known 5 days later [3] then leading to expensive and complicated remediation procedures.

Other problems with the method that have been reported are (1) the need to dilute samples, given that low solubility of oxygen in water (8.7 mg L^{-1} at 25 °C) quickly becomes the rate-limiting reagent (the linear range of the method is between 1 and 9 mg L^{-1} of O_2 or mg L^{-1} BOD_5); (2) the lack of stoichiometric validation [4]; and (3) temperature sensibility [5]. Despite these limitations, the BOD_5 test is used extensively and recognized by regulatory agencies.

Alternatively biological approaches applied in conjunction with chemical analysis can give more complete information about a potential biological impact produced by complex and polluted effluents. The most relevant biological techniques applied to environmental analysis can be classified as bioassays, immunoassays or biosensors according to the technical principles used [6].

Bioassays and biosensors are biological tools used for measuring a global biological effect on living organisms or their component

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parts, as well as for assessing global parameters such as BOD. The difference between both tools lies in the disposition of the biological recognition element that in the case of biosensors is immobilized on a transducer [6].

Karube proposed in 1977 a BOD biosensor based on immobilized whole cells and an oxygen electrode [5] to overcome the major problem (the 5 days determination time) of the aforementioned BOD_5 method. BOD biosensors usually measure the respiration rate of microorganisms at an oxygen electrode interface that correlates with biodegradable material [7].

However biosensors with immobilized microorganisms based on membrane entrapment can yield non-reproducible results given the vital role played by changes in their physiological state and the concentration on the membrane [8–17]. Biofilm-based systems also have been proposed, but problems related mainly to storage and preservation conditions remain [18].

Microbial sensors are perhaps best suited for rapid determination of BOD if the microbial system selected is capable of rapidly degrading a wide range of organic compounds. There are two main options that have been proposed: the use either of a microbial consortium or a unique strain with a broad range of degradation capacity. To achieve utilization of a broad range of organic compounds many different microorganisms could be mixed [5,7,12,13,19,20]; however, it has been shown that mixed community biosensors change their properties in time leading to non-reproducible results [7,13].

To avoid this limitation we sought to design and construct a microbial bioassay based on a unique strain, thus resulting in a more stable behavior, even though a single strain usually does not metabolize a broad range of organic compounds.

The genus *Klebsiella* is defined as a Gram negative, nonmotile, lactose-fermenting, usually encapsulated rod-shaped bacteria of the *Enterobacteriaceae* family, ubiquitous in nature [21–23]. Almost all *Klebsiella* strains grow readily in ordinary media and also in minimal medium, with ammonium ions or nitrate as sole nitrogen source and without a requirement of growth factors. Such bacteria have shown to utilize all the major sugars, aminoacids, and uronic acids derived from the hydrolysates of hemicellulosic and cellulosic materials [21,24]. Many environmental strains have been found involved in degradation of herbicide, pesticide, and several other toxic compounds [25–30]. Therefore *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the biotypes more adequate for a bioassay design and also present less nutritional requirements [21].

Even though the vast majority of biosensors and bioassays for BOD_{st} determination are based on amperometric oxygen electrodes [8,9] (or recently, oxygen optrodes) [31–33] as transducer system, these approaches cannot overcome the main methodological drawback of low oxygen solubility in water.

To overcome the abovementioned drawback a ferricyanide-mediated BOD_{st} technique has been reported as an approach in which O_2 was replaced by potassium hexacyanoferrate (III) (ferricyanide) [34]. Ferrocyanide, the soluble reduced mediator, is accumulated while organic compounds are microbially degraded producing carbon dioxide, as shown in Eq. (1) [19].

$$\{CH_2O\} + H_2O + 4\,Fe(CN)_6^{3-} \rightarrow CO_2 + 4\,Fe(CN)_6^{4-} + 4\,H^+ \qquad (1)$$

The ferrocyanide quantification, conducted by amperometry or coulometry, has been used as an indirect method to determine BOD_5 values [10,11,19,20,35,36].

MICREDOX®, a ferricyanide-mediated BOD_{st} assay based on coulometric measurements in which *Proteus vulgaris* is incubated for 1 h, has improved the linear range of detection (between 18.8 and 150 mg L^{-1} BOD_5) without the need of sample dilution. Although some advances have been made using PVA-immobilized bacteria, the preceding assay cannot yet be transformed easily into a portable instrument, given the need of de-aerate samples [11].

Furthermore the centrifugation step necessary to stop the microbial reaction has not yet been avoided in ferricyanide-mediated methods where free living bacteria are used as catalysts [19,20,36–38]. The development of a solution to this problem is critical since current measurements are necessarily consecutive (for a simple non-multiplexed potentiostat) and, if bacteria activity is not stopped, ferricyanide reduction continues while samples are being measured. So there is a real need to standardize time incubations, i.e. stop the biochemical reaction at the same time in all the simultaneously assayed samples, before the measurement is done.

Despite the fact that centrifugation is the method commonly used to stop microbial ferricyanide reduction it is not a practical resource for an autonomous portable device. We then present a ferricyanide mediated BOD assay where the critical issue resolved is standardization of incubation times in all samples by using a bacteria growth inhibitor to stop microbial ferricyanide reduction.

Many compounds usually employed as disinfectants involve a relative lack of selective toxicity [39]. If the inhibitor compound is effective in small quantities, the employment of a disinfectant may be the solution to avoid centrifugation. So we assayed ordinary compounds with known inhibitory properties in bacterial metabolism and low cost, such as formaldehyde, iodine povidone, sodium hypochlorite, sodium azide, chloroxylenol, ethanol, isopropanol, thymol, H_2O_2 and NaOH [39–41].

There is another drawback in the ferricyanide-mediated BOD technique related to the effect that the cyanide ions released by ferricyanide may cause in the performance of the bioassay. Ferricyanide could disrupt electron transfer to itself due to the liberation of free cyanide anions. In this regard, Liu has proved that ferricyanide may produce growth inhibition, respiration decrease and morphological changes in *E. coli* and that those effects were not reversible depending on the ferricyanide concentrations used [42,43].

We present here improvements to the ferricyanide-mediated approach with a cell-based bioassay in which *K. pneumoniae* (referred to here as $BOD_{K.\ pneumoniae}$) has been successfully used to determine the BOD values of 4 municipal wastewater and 2 synthetic samples; and for the first time we propose the use of formaldehyde as an inhibitor to successfully stop the microbial ferricyanide reduction (leading to the standardization of incubation times) without affecting the amperometric technique.

2. Materials and methods

2.1. Solutions and culture media

The LB broth contained (g L^{-1}) bacto triptone (10), NaCl (10), and yeast extract (5). For agar plate count, 1.5% (w/v) of agar was added.

The minimum medium (MM) contained (g L^{-1}) Na_2HPO_4 (6), KH_2PO_4 (3), NH_4Cl (1), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.12), and $CaCl_2{\cdot}2H_2O$ (0.01), with pH adjusted to 7. The potassium ferricyanide stock solution (100 mmol L^{-1}) was prepared in MM.

The D-glucose, D-fructose, L-glutamic acid, lactose, succinic acid, sucrose and GGA solutions were made in MM and sterilized by membrane filtration (0.22 μm).

The OECD synthetic wastewater contained (g L^{-1}) peptone (15), meat extract (11), urea (3.0), NaCl (0.7), $CaCl_2$ anhydrous (0.3), $MgSO_4{\cdot}7H_2O$ (0.2), and $K_2HPO_4{\cdot}3H_2O$ (3.7) with a measured BOD_5 value of 17,000 mg L^{-1}.

The synthetic sample Sim-1 final concentration in well (mg L^{-1}) was: peptone (11), meat extract (150) in MM. A 1:2 dilution of Sim-1 (Sim-2) was also assayed.

The inhibitor compounds employed and the corresponding stock solution concentrations were: chloroxylenol (48 g L^{-1} from